ABC-F proteins mediate antibiotic resistance through ribosomal protection

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Introduction

Elucidation of the mechanisms by which bacteria resist the inhibitory effects of antibiotics provides essential intelligence in the ongoing fight against antibiotic resistance. Whilst the majority of clinically important antibiotic resistance mechanisms are by now well characterised, some key gaps in our knowledge remain. One such gap concerns the mechanism by which ABC-F proteins mediate resistance to antibiotics that target protein synthesis in Gram-positive pathogens; although members of the ABC-F family collectively yield resistance to a broader range of clinically important antibiotic classes than any other family of resistance determinants, their mechanism of action has been the subject of controversy since their discovery at the University of Leeds 25 years ago.

ABC-F proteins comprise a single polypeptide containing two ATP-binding cassette (ABC) domains separated by a linker of ~80 amino acids. In contrast to canonical ABC transporters, the ABC portions of ABC-F proteins are not fused to transmembrane domains (TMDs), nor are they genetically associated with TMDs in operons. Two competing hypotheses have been proposed to explain how these proteins mediate resistance to antibiotics (Figure 1). The

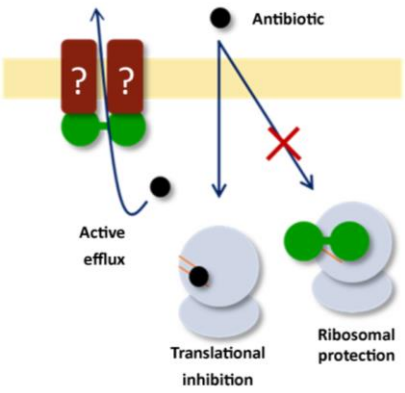

Figure 1: Proposed mechanisms of ABC-F mediated antibiotic resistance. In an antibiotic-susceptible bacterium, translation is inhibited by the antibiotic, leading to growth arrest (centre). ABC-F proteins (green) are proposed to rescue translation from inhibition either by recruiting an unknown membrane-spanning protein to promote active efflux of the antibiotic (left), or, by displacing the antibiotic from its ribosomal binding site (right).

efflux hypothesis posits that ABC-F proteins associate with as-yet-unidentified TMDs to form a functional efflux complex capable of exporting antibiotics out of the cell, whilst the ribosomal protection hypothesis suggests that these resistance proteins act instead to reduce the accessibility or affinity of antibiotic binding sites on the 50S subunit of the ribosome, thereby directly protecting the translational machinery from antibiotic-mediated inhibition. This project was initiated with the intention of elucidating the mechanism of antibiotic resistance mediated by ABC-F proteins.

Results

ABC-F proteins protect staphylococcal translation from antibiotic inhibition *in vitro:* We initially sought to determine whether ABC-F proteins could mediate antibiotic resistance *in vitro*, under conditions where efflux was not possible. To this end, staphylococcal S30 extracts were generated, and used to establish *in vitro* transcription-translation (T/T) assays. Two phylogenetically-distant ABC-F proteins, Vga(A) and Lsa(A), were heterologously expressed in *E. coli*, purified to homogeneity, and tested for their ability to protect T/T assays from antibiotic inhibition. Both proteins were shown to mediate dose-dependent restoration of translational activity (Figure 2A).

To provide confirmation that the observed ability of ABC-F proteins to protect *in vitro* translation from antibiotics reflected the activity of these proteins in whole cells, we sought to recapitulate in the T/T assay three phenotypes that have been associated with these proteins in bacteria: i) The Vga(A) protein is not functional in *E. coli*, failing to confer any reduction in virginiamycin M susceptibility even when detectably overexpressed. This result was mirrored in an *in vitro* T/T assay using *E. coli* S30 extract. ii) It has previously been demonstrated that substitution for glutamine of the catalytic glutamate residue following the Walker B motif in either ABC domain of Vga(A) results in a non-functional protein incapable of mediating antibiotic resistance. We confirmed that this also holds true in *in vitro*, with addition of purified Vga(A)$_{E105Q}$ to a T/T assay using *S. aureus* S30 extract producing no restoration of translation activity. iii) A single amino acid substitution ($K_{219}T$) in the linker region between the two nucleotide binding domains of Vga(A) has been reported to increase the level of phenotypic resistance to antibiotics of the lincosamide class from low-level to high-level. This shift in resistance profile was successfully recapitulated in the *S. aureus* T/T assay; addition of purified Vga(A)$_{K219T}$ to a T/T reaction inhibited with a lincosamide resulted in restoration of translation activity, whilst wild-type Vga(A) did not detectably protect translation against the drug.

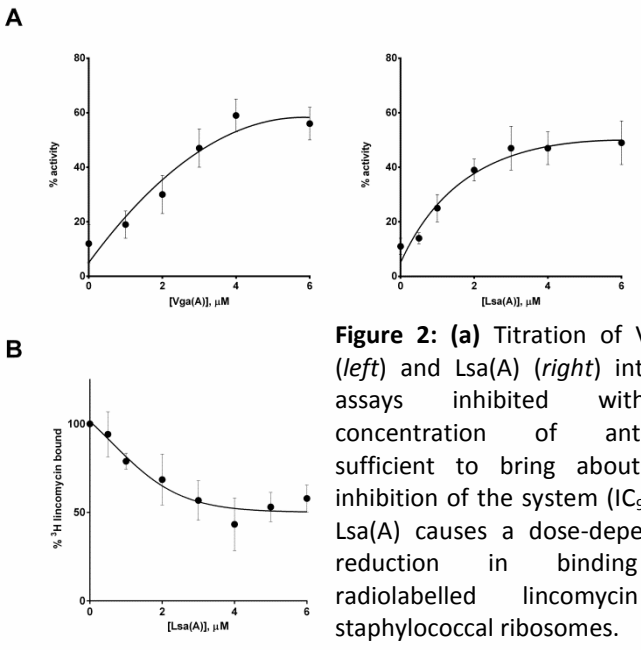

Figure 2: (a) Titration of Vga(A) (*left*) and Lsa(A) (*right*) into T/T assays inhibited with a concentration of antibiotic sufficient to bring about 90% inhibition of the system (IC_{90}). **(b)** Lsa(A) causes a dose-dependent reduction in binding of radiolabelled lincomycin to staphylococcal ribosomes.

ABC-F proteins directly displace antibiotics from the ribosome: To assess whether ABC-F proteins protect the translation apparatus from antibiotic-mediated inhibition by directly interfering with the interaction between the antibiotic and the ribosome, we evaluated the ability of the Lsa(A) protein to prevent binding of radiolabeled (^{3}H) lincomycin to purified staphylococcal ribosomes. Pre-incubation of Lsa(A) with ribosomes prior to the addition of ^{3}H-lincomycin resulted in a dose-dependent decrease in subsequent binding of the drug to ribosomes (Figure 2B). Subsequently, we also examined the ability of Lsa(A) to displace pre-bound ^{3}H-lincomycin from ribosomes, establishing that addition of an 8-fold molar-excess of Lsa(A) to ribosomes pre-incubated with drug resulted in a substantial (~73%) reduction in ribosome-associated ^{3}H lincomycin.

Collectively, our results show that the ABCF-F proteins mediate antibiotic resistance through ribosomal protection. Future studies will focus on gaining insights into the structure and molecular mechanism of these proteins.

Funding
This work was funded by the BBSRC.